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A Flow Cytometric Assay for the Study of E3 Ubiquitin Ligase Activity^b

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Abstract

Background—Current methods for monitoring E3 ubiquitin ligase activity in cell culture or in vivo are limited. As a result, the degradation of cellular targets by many E3 ubiquitin ligases in live cells has not yet been examined.

Methods—A target of an E3 ubiquitin ligase was expressed as a fluorescently labeled protein in cell culture. If the E3 ubiquitin ligase mediates the degradation of a target protein in cell culture, it is expected that the target will show a reduced fluorescence signal by FCM analysis. We initially used the E3 ubiquitin ligase, herpes simplex virus type 1 (HSV-1) infected cell protein 0 (ICP0) and one of its targets, promyelocytic leukemia (PML) protein, to determine the feasibility of our approach. Cells expressing a PML-GFP fusion protein were selected by cell sorting and infected with an adenoviral vector expressing ICP0.

Results—In contrast to mock-infected cells, only PML-GFP-expressing cells infected with the ICP0 adenoviral vector led to a significant decrease in the fluorescence signal of PML-GFP when examined by fluorescence microscopy and FCM analysis.

Conclusions—Using HSV-1 ICP0 as a paradigm, it is possible to examine the live activity of an E3 ubiquitin ligase (via one of its targets) in cell culture with FCM analysis.

Keywords

GFP; herpes simplex virus type 1; infected cell protein 0; E3 ubiquitin ligase; promyelocytic leukemia; FACS

Examining the E3 ubiquitin ligase activity in live mammalian cells has proven challenging, as most methods to examine this activity result in the loss of cell viability. Thus, there is a need to develop a simple and sensitive assay to monitor and quantify this activity in real time. E3 ubiquitin (Ub) ligases are cellular and pathogenic proteins that play pivotal roles in diverse cellular processes (Reviewed in (1)). Typically, E3 Ub ligases are components of distinct biochemical pathways that attach and polymerize Ub (a 76 amino acid post-translational modification) to target proteins, marking them for degradation by the proteasome. Genetic, cell biology, and biochemical studies have demonstrated that E3 Ub ligases play key roles in controlling cellular events such as proliferation, apoptosis, and viral replication. The process of ubiquitination requires several proteins that must sequentially

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coordinate their activities in a specific pathway. Ubiquitination begins when an E1 Ub activating enzyme (Uba) forms a bond with Ub and then transfers Ub to an E2 Ub conjugating enzyme (Ubc). In most instances, interaction of the E2 Ubc with an E3 Ub ligase is required to target specific proteins for degradation. E3 Ub ligase proteins or complexes recognize target proteins and catalyze the transfer of Ub from the E2 Ubc to form polymers on these targets. These polyubiquitinated proteins are recognized by the proteasome and consequently degraded.

One E3 Ub ligase that has been recently characterized is herpes simplex virus type 1 (HSV-1) infected cell protein 0 (ICP0). ICP0 is expressed directly after viral infection (2,3) and is a potent and global transcriptional transactivator of viral and select cellular genes (4–8). Although ICP0 is not absolutely required for viral replication in culture, it confers a significant growth advantage to the virus, especially at low multiplicities of infection (9–12). When expressed in cultured cells, ICP0 localizes to and disrupts nuclear structures termed nuclear domain (ND) 10, which are also known as promyelocytic leukemia or PML oncogenic domains (PODs) or nuclear bodies (NBs) (13,14). The ICP0-mediated disruption of ND10 structures correlates with decreased levels of ND10-associated antigens and the degradation of distinct cellular proteins via the ubiquitin-proteasome pathway (10,15–18). Degradation of these proteins can be explained, in part, by recent studies demonstrating that the RING finger of ICP0 confers E3 Ub ligase activity on it, and this activity mediates the selected proteolysis of cellular proteins including PML (19,20). Two additional cellular proteins targeted for ICP0-directed degradation via the proteasome pathway are the catalytic subunit of DNA dependent protein kinase (DNA-PK_{cs}) (21), which plays a role in the repair of DNA double-strand breaks (22), and ubiquitin specific protease 7 (23), which removes Ub from proteins or breaks down Ub polymers. Notably, the disruption of ND10 structures and the degradation of cellular proteins via the ubiquitin-proteasome pathway also appear to play a central role in the transcriptional transactivating activity of ICP0 (10), linking the transactivating activity of ICP0 with its protein degradative activity.

The activity of E3 Ub ligases in cell culture is often monitored by the destruction of target proteins using immunofluorescence, immunoprecipitation, and/or immunoblot analyses (24–26). The limitation of these assays is that it is not possible to examine the real time (live) activity of E3 Ub ligases because these cultures must be lysed or fixed. Alternative approaches to examine the functions of proteins in living cells are based on protein-protein interaction assays such as yeast two-hybrid (YTH), fluorescence resonance energy-transfer (FRET), reporter protein complementation/reconstruction, bimolecular fluorescence complementation (BiFC), and two-hybrid protein translocation biosensor (PTB) system (27–29). Notably, these approaches require the expression and simultaneous detection of at least two proteins, which is challenging with E3 Ub ligases because they typically direct the degradation of their targets. In a previous study, the activity of the E3 Ub ligase, ICP0, was studied by fluorescence microscopy in live cells that expressed a fluorescently labeled target protein (30). While this method showed that ICP0 mediates the degradation of one of its targets, the extent of protein degradation per cell and over a whole cell population was not quantified. Thus, the limitations of these assays demonstrate a need for a method to rapidly and sensitively monitor the activity of an E3 Ub ligase in viable cell cultures.

The goal of this report is to develop a novel strategy to examine the activity of E3 Ub ligases in live cells. To monitor this activity, targets of these ligases will be expressed as fluorescently labeled proteins in cell culture. If an E3 Ub ligase mediates the degradation of its target in culture, it is expected that the target protein will show a reduced fluorescence signal in the presence of the ligase as measured by flow cytometry. Because ICP0 is a known E3 Ub ligase and a small group of its targets have been identified, we initially used ICP0 and one of its targets, PML fused to green fluorescent protein (GFP), to determine the

feasibility of our approach in live mammalian cells. Our results indicate that we can rapidly examine the live degradative activity of an E3 Ub ligase by FCM analysis.

MATERIALS AND METHODS

Establishment of WI-38 cells infected with the retrovirus vector, pMX-GFP or pMX-PML-GFP

Human embryonic lung (WI-38) cells obtained from the American Type Culture Collection (ATCC; Manassas, VA) were cultured in Alpha Minimum Essential Medium (AMEM) supplemented with 10% fetal calf serum, penicillin-streptomycin solution, and L-glutamine. Cells were infected with the retroviral vector pMX-GFP, at 0.3 fluorescence forming units (FFU) per cell or mock-infected for 48 hours (h). pMX-GFP, developed by Kitamura and colleagues (31), was packaged as a retroviral vector using the Pantropic Retroviral Expression System (Clontech, Mountain View, CA) according to the manufacturer's protocol. Live GFP-expressing cells were sorted for establishment of WI-38+GFP cells, which stably express GFP, using a BD FACS Aria cell sorter (BD Biosciences, San Jose, CA) at the University of Kansas Medical Center Flow Cytometry Core Facility. Performing the same procedures described above, WI-38 cells were also infected with pMX-PML-GFP, a retroviral vector that stably expresses the first 539 amino acids of PML fused to GFP, allowing for the establishment of WI-38+PML-GFP cell cultures.

Preparation of adenoviral stock

ICP0 was expressed in cells using the adenoviruses Ad.T-ICP0 and Ad.C-rtTA, which inducibly express ICP0 from a system developed by Gossen and Bujard (32). In this system, doxycycline (Dox) activates the reverse tetracycline transactivator rtTA (expressed from Ad.C-rtTA) to initiate transcription from the tetracycline response element (TRE) promoter of the ICP0 gene in Ad.T-ICP0 (33). Ad.C-rtTA, Ad.T-ICP0, and Ad.T-n212, which expresses a mutant form of ICP0, were grown and titered as previously described (33).

Immunofluorescence staining of DNA-PK_{cs}, p21, and ICP0 in WI-38 cells

To establish the inducibility of our adenoviral vector by degrading a known cellular target of ICP0, endogenously expressed DNA-PK_{cs}, WI-38 cells were plated in a 24-well plate for 24 h and then co-infected with 3 plaque forming units (PFU) per cell of Ad.T-ICP0 and 40 PFU per cell Ad.C-rtTA in the presence or absence of 12 μM Dox (Sigma, St. Louis, MO) for 12 h. Cells were then fixed, permeabilized, and incubated with DNA-PK_{cs}-specific (sc-5282; Santa Cruz Biotechnology, Santa Cruz, CA) and ICP0-specific antibodies (J17), and these antibodies were detected with a FITC-conjugated antibody (Jackson ImmunoResearch, West Grove, PA), and a Rhodamine-Red X conjugated antibody (Jackson ImmunoResearch, West Grove, PA), respectively, as previously described (34). Cells were viewed by fluorescence microscopy at X400 magnification using a Nikon Eclipse TE2000-U and a X-Cite 120 XL illumination unit with a high pressure 120 Watt metal halide short arc lamp. The FITC filter excites at 450–440 nm and emits at 515–555 nm. The Texas red filter excites at 532–587 nm and emits at 608–683 nm. Cells were photographed with a digital camera. As a negative control for these studies, immunofluorescence staining conditions for the cellular protein, p21, was carried out as described previously (35), except that 4% paraformaldehyde was used and primary antibody incubations were carried out for 60 min. An ICP0-specific antibody (J17) was detected with a Rhodamine-Red X conjugated antibody as above, and a p21 specific antibody (WAF-1 (Ab-1); Oncogene, Cambridge, MA) was detected with alexafluor 488 (Invitrogen Corporation, Carlsbad, CA).

Examination of PML-GFP fluorescence by microscopy and FCM analysis

i) Microscopy—WI-38+PML-GFP cells were plated on rat tail collagen-coated coverslips placed in 12-well plates (3×10^4 cells per well) and 24 h later were either mock-infected, or infected with 10 PFU per cell of Ad.T-ICP0 or the negative control Ad.T-n212, and Ad.C-rtTA in the presence of 12 μ M Dox for 1h. Cells were washed 3 times with Dulbecco's Phosphate-Buffered Saline (dPBS) and AMEM containing 12 μ M Dox was added. Infections were allowed to proceed for 24 h, and coverslips were washed with dPBS, and inverted on microscope slides containing dPBS. Live cells were examined by fluorescence microscopy at X400 magnification as before and photographed with a digital camera.

ii) FCM analysis—Concurrent with our microscopy studies, cells were also analyzed by flow cytometry. Included in this experiment were WI-38 cells not transduced with a GFP-retrovirus expression vector as a negative control. Cells were plated at 5×10^5 cells per 60 mm dish, and 24 hours post infection (hpi), single cell suspensions were prepared by trypsinization, and fluorescence levels were determined by FCM to monitor PML-GFP expression. FCM analysis was performed on a FACScan Flow Cytometer (BD Biosciences, San Jose, CA) with a 488 nm argon-ion laser and bandpass filters of 530/30 nm for GFP. The results were analyzed using CellQuest software (BD Biosciences, San Jose, CA).

Sorting and analysis of WI-38+PML-GFP/WI-38+GFP mixed cell populations expressing ICP0

To determine ICP0 target specificity, WI-38+PML-GFP and WI-38+GFP cells were mixed in various ratios, and ICP0 was expressed in these cells. Ratios of WI-38+PML-GFP to WI-38+GFP were 90:10, 10:90, and 1:99, respectively, and pure populations of each cell type were used in the mock-infected control samples. Infected cultures were plated at 1×10^7 cells per T150 flask, and mock-infected cultures were plated at 1×10^6 cells per T75 flask. Because the PML-GFP-expressing cells represented 1% of the PML-GFP/GFP population in one of our mixed cell samples, it was necessary to plate a greater number of infected cells than mock-infected cells to achieve sufficient sorted cell numbers to carry out the microscopic analyses described below. After 24 h, cells were infected with 8 or 20 PFU per cell Ad.C-rtTA and Ad.T-ICP0 in the presence of 12 μ M Dox. 24 hpi, cells were trypsinized, pelleted at $200 \times g$ for 3 min., washed with dPBS, repelleted, and resuspended in 1 mL AMEM. Infected cultures were stained with PI (to exclude non-viable cells in our experiments), and all samples were sorted using a BD FACSAria cell sorter (BD Biosciences, San Jose, CA) with an 488 nm argon for excitation and bandpass filters of 530/30 nm for GFP and 576/26 nm for PI. Sorts were analyzed with BD FACSDiva software (BD Biosciences, San Jose, CA) at the University of Kansas Medical Center Flow Cytometry Core. Low-expressing-GFP cells (with a signal less than PML-GFP in the absence of ICP0) and GFP(−) cells were selected by cell sorting and pooled, pelleted at $200 \times g$ for 3 min., resuspended in AMEM, and plated on a rat tail collagen-coated 24-well glass bottom plate with a coverslip thickness of 0.16–0.19 mm (MatTek Corporation, Ashland, MA) for 24 h in the absence of Dox, to turn-off ICP0 expression and restore PML-GFP fluorescence. For imaging, cells were washed 3X with dPBS and placed in phenol-red free Dulbecco's Modified Eagles Medium (Invitrogen, Carlsbad, CA). The sorted cells were viewed by fluorescence microscopy as described before, and the number of WI-38+GFP and WI-38+PML-GFP cells was counted per sample relative to the total number of sorted cells counted to determine the ratio of true-to false-positives selected.

RESULTS

ICP0 expressed from an adenovirus mediates the degradation of DNA-PKcs

We sought to determine the efficiency and inducibility of ICP0 expression and its E3 Ub ligase activity from a set of non-replicating adenoviral expression vectors in WI-38 cells. This adenoviral vector system will be used to monitor the degradation-directed activity of ICP0 in live cells. As previously mentioned, ICP0 is known to induce the degradation of DNA-PK_{cs} by the ubiquitin-proteasome pathway, which was established using a proteasome inhibitor (21). Thus, we initially used endogeneously expressed DNA-PK_{cs} as a cellular target to monitor ICP0's E3 ubiquitin ligase activity. In addition, we wanted to exclude the possibility that the adenoviral vectors themselves could degrade DNA-PK_{cs}. In these tests, WI-38 cells were co-infected with Ad.T-ICP0 and Ad.C-rtTA in the presence or absence of Dox for 12 h. This adenovirus expression vector system allows us to turn on and off ICP0 expression, as described in the Materials and Methods. WI-38 cells were chosen for these studies because known cellular targets of ICP0-mediated degradation are readily observed in these cells. Twelve h after co-infection, cells were fixed, permeabilized, and incubated with DNA-PK_{cs}-specific and ICP0-specific antibodies to examine the expression of DNA-PK_{cs} and ICP0 by fluorescence microscopy.

As shown by the fluorescent staining patterns in Figure 1, the presence (+) of Dox induced the efficient expression of ICP0, whereas its expression was undetectable in the absence (–) of Dox. Also, ICP0 induced the degradation of DNA-PK_{cs} as observed by the absence of cells exhibiting colocalization of ICP0 and DNA-PK_{cs} in the presence of Dox. Furthermore, we did not observe the degradation of DNA-PK_{cs} in the absence of Dox, demonstrating that ICP0 expression was tightly regulated and that the adenoviral vectors themselves did not induce the degradation of DNA-PK_{cs}. As a negative control for ICP0-directed degradation in this system, the immunofluorescence staining of the nuclear protein, p21, an inhibitor of CDKs, was unaffected by ICP0 expression in WI-38 cells (see Fig. S1). These results demonstrate the feasibility of using these adenoviral vectors as a source of ICP0 to direct the degradation of cellular protein targets.

ICP0 reduces the fluorescence of PML-GFP by microscopy and flow cytometry

The goal of this study is to examine the live activity of an E3 Ub ligase in cell culture. In addition to DNA-PK_{cs}, it is well established that PML, an ND10-associated protein, is also a target of ICP0-directed degradation. It was unclear whether a known target of ICP0-directed degradation would be efficiently degraded when expressed exogenously as a GFP fusion protein in developing our system. To address this concern, WI-38 cells were infected or transduced with a retroviral vector that expresses a PML-GFP fusion protein, and PML-GFP-expressing cells were selected by FACS. Aliquots of these pooled cells were either mock-infected or infected with non-replicating adenoviral vectors that inducibly express wild-type ICP0 (Ad.T-ICP0) or a mutant allele of ICP0, *n212* (Ad.T-*n212*; 30), which was used as a negative control for ICP0-directed degradation, and the rtTA transcription factor (Ad.C-rtTA) in the presence of Dox. Infections were allowed to proceed for 24 h, and cells were examined by fluorescence microscopy. The results of this experiment are shown in Figure 2. Only those cells infected with the Ad.T-ICP0 expression vector led to a significant decrease in the fluorescence signal of PML-GFP, in contrast to mock-infected or Ad.T-*n212*-infected cells. Thus, ICP0 directs the degradation of a known cellular target, PML, when PML was expressed as a GFP fusion protein in WI-38 cells as determined by fluorescence microscopy.

To confirm that the reduction in the PML-GFP signal mediated by ICP0 was detectable by FCM, infections were carried out as described above. An additional sample, WI-38 cells that

were not transduced with a GFP-retroviral expression vector, was included in this study to control for the background level of fluorescence in the absence of GFP. At 24 hpi, cells were harvested, single cell suspensions were prepared by trypsinization, and FCM analysis was performed to monitor GFP expression. The results of this experiment are shown in Figure 3. As observed with fluorescence microscopy (Fig. 2), only those cells infected with the Ad.T-ICP0 inducible expression vector led to an apparent decrease in the GFP signal of the PML-GFP peak compared to mock-infected or Ad.T-n212-infected cells. This decrease, however, did not reach the background level of fluorescence originating from the parental WI-38 cells. This latter observation suggests that low levels of PML-GFP are present in Ad.T-ICP0-infected cells, likely resulting from a high level of expression of the fusion protein in our experimental system. Nevertheless, these results demonstrate that flow cytometry can be used to monitor the degradative activity of an E3 Ub ligase.

PML-GFP cells are selected for in PML-GFP and GFP mixed cell populations containing ICP0 by FCM analysis

After detecting the ICP0-mediated degradation of PML-GFP, we next sought to determine the specificity of selecting PML-GFP expressing cells as our E3 Ub ligase target. To address the issue of specificity, we performed experiments by mixing different amounts of PML-GFP and GFP cells and induced them to express wild-type ICP0 with the adenoviral vectors. Our FCM analysis in Figure 4 showed that regardless of the mixed cell population examined, there was a significant shift in the mean fluorescence intensity (MFI) of the PML-GFP peak but not the GFP peak, when compared to their mock-infected counterparts in each mixed population. The PML-GFP and GFP peaks were identified by the distinct fluorescence signals of the individually sorted cell populations (see Fig. S2). For mixed cell populations of 90% PML-GFP/10% GFP, 10% PML-GFP/90% GFP, and 1% PML-GFP/99% GFP, the MFI shifted from 2,100 to 560; 2,400 to 660; and 4,000 to 800, respectively, for the PML-GFP peak. Pure populations of mock-infected PML-GFP- and GFP-expressing cells (Fig. S2) show that these cell types have distinct fluorescent signals. From these sorts, we took isolated cell populations from the “collected” gates, with fluorescence signals that were less than those of mock-infected PML-GFP cells. We expected these isolated cell populations to contain our ICP0 target (i.e., PML-GFP), because they exhibited diminished fluorescence by cell sorting. The resulting collected cell populations were plated in the absence of Dox, to turn off ICP0 expression, and examined by fluorescence microscopy. As shown in Figure 5, our target cells containing PML-GFP were preferentially selected. In a population expressing 90% PML-GFP and 10% GFP cells in the presence of ICP0, practically all sorted cells (99.99%) expressed PML-GFP (see postsort, Fig. 5). For a cell population expressing 10% PML-GFP and 90% GFP, postsort cells were overwhelmingly PML-GFP (99.9%). For a cell population containing 1% PML-GFP and 99% GFP cells, there was a significant increase (82%) in the percentage PML-GFP cells. We observed similar trends (98.3%, 83.6%, and 51.2%) for cell populations containing 90%, 10%, and 1% PML-GFP using independent preparations of adenoviral stocks. Taken together, our results indicate that ICP0 directs the selective degradation of PML-GFP in live cells.

DISCUSSION

While the degradative activity of E3 Ub ligases has been studied using immunoprecipitation/Western blot assays or fluorescence microscopy, quantitatively examining their activity in live cells has been problematic because these procedures require cell lysis or fixation (24–26). Current methods that rely on monitoring the live activities of proteins in cells are largely based on protein-protein interactions (e.g., YTH, FRET, reporter protein complementation/reconstruction, BiFC, and PTB; 27–29). These systems are not suitable for studying E3 Ub ligases as they rely on the expression of all labeled proteins for these assays

to function, whereas E3 Ub ligases typically direct the degradation of their targets. One report examined the live activity of an E3 Ub ligase in tissue culture by fluorescence microscopy, but quantitative analysis of target protein degradation in individual cells and over a cell population was not performed (30). Notably, our results in this report propose FCM analysis as a new approach to semi-quantitatively monitor the activity of E3 Ub ligases in living cells.

To develop our method by flow cytometry, we used the E3 Ub ligase, HSV-1 ICP0, and cells that expressed a GFP-tagged version of one of its targets, PML. We were able to show that ICP0 reduced the fluorescence of nuclear PML-GFP in live cells, an indication of its degradation, by fluorescence microscopy (Fig. 2) and FCM analysis (Figs. 3 & 4). This decrease in fluorescence could be quantified by examining the MFI of a given cell population (Fig. 4). Cell mixing experiments demonstrated that our sorted cells (PML-GFP) were preferentially targeted for degradation in our selection procedure (collected low GFP or GFP(−) cells, Figs. 4 & 5). The advantages of this approach over immunoprecipitation/immunoblot and immunofluorescence assays are that the degradative-inducing activity of E3 ligases can be examined in live cells, that the samples require limited handling, which can be rapidly and semi-quantitatively analyzed (within 1–2 hours), and that performing the assay does not depend upon the generation of antibodies. As indicated in Fig. 5, additional benefits of this method are that target cells survive the sorting procedure and can be placed in culture and reassayed or used in further analyses.

Potential shortcomings of our approach include the range of cell types that can be analyzed, as they must be permissive for adenoviral vector entry and retroviral vector integration; establishing that properties or function of a target protein of interest is unaffected when the protein is fused to GFP or its derivatives; difficulties in studying toxic target proteins or those that inhibit cellular proliferation; and controlling the levels of an exogenously expressed target protein to ensure that it is efficiently degraded in the presence of the E3 Ub ligase. Many of these difficulties can be overcome by using alternate strategies. For example, should retroviral vector integration be impaired for a specific cell type (i.e., post-mitotic cells), utilizing other retroviral vectors (i.e., lentiviral vectors) would alleviate this problem. Should a fluorescent tag inadvertently affect an activity of a target protein, changing the placement of the tag (e.g., from the C- to the N-terminus or internally) would, in many instances, permit a given target protein to function properly. Issues related to target protein toxicity and the efficient degradation of a target protein could be controlled with an inducible retroviral vector expression system. Given these shortcomings and optional approaches, preliminary tests should quickly determine the feasibility of using this novel system for a specific ligase-target pairing.

The development of this system will facilitate structure/function, kinetic, and signal transduction analyses of proteolytic proteins and their targets, allowing one to understand the at the molecular level how these proteins regulate numerous cellular processes. Furthermore, it may be possible to use this approach to identify proteins whose degradation is mediated by proteolytic enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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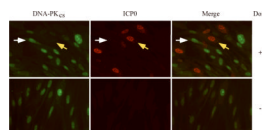


Figure 1. ICP0 degrades endogenous DNA-PKcs

WI-38 cells were infected with 3 PFU per cell of Ad.T-ICP0 and 40 PFU per cell Ad.C-rtTA for 12 h in the presence (+) or absence (–) of the rtTA inducer, Dox, as indicated on the right of the figure. At 12 h post infection, cells were washed, fixed, permeabilized, and incubated with primary antibodies against DNA-PK_{cs} and ICP0. The DNA-PK_{cs} and ICP0 primary antibodies were detected with a FITC-conjugated antibody and a Rhodamine-conjugated antibody, respectively. Monolayers were observed by fluorescence microscopy for endogenous DNA-PK_{cs} (left column), ICP0 expressed from the adenoviral vector (middle column), and the composite of the two images (merge, right column). The white arrow indicates a cell positive for DNA-PK_{cs} staining but negative for ICP0 staining, and the yellow arrow indicates a cell positive for ICP0 staining but negative for DNA-PK_{cs} staining. The white bar at the bottom right is 50 μ m.

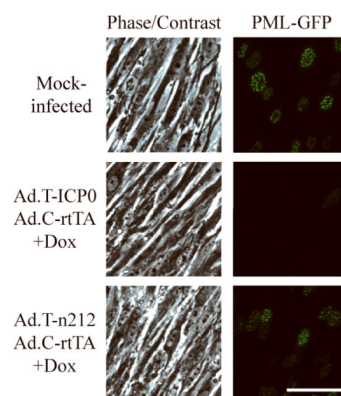


Figure 2. ICP0 diminishes PML-GFP fluorescence as observed by microscopy

WI-38 cells that express PML-GFP were mock-infected or infected with 10 PFU per cell of Ad.T-ICP0 or Ad.T-n212 and 10 PFU per cell of Ad.C-rtTA for 24 h in the presence of Dox, as indicated on the left of the figure. At 24 h post infection, cells were washed, and monolayers were viewed by phase/contrast (left column) and fluorescence microscopy (right column). The white bar at the bottom right is 50 μ m.

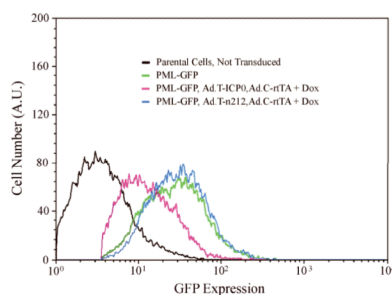


Figure 3. ICP0 reduces PML-GFP fluorescence as detected by FCM analysis

WI-38 cells that do not express GFP (Parental Cells) or express PML-GFP were mock-infected or infected with 10 PFU per cell of Ad.T-ICP0 or Ad.T-n212 and 10 PFU per cell of Ad.C-rtTA for 24 h in the presence of Dox. At 24 h post infection, cells were trypsinized into single cell suspensions and examined by FCM analysis. A minimum of 20,000 events was scored for each sample.

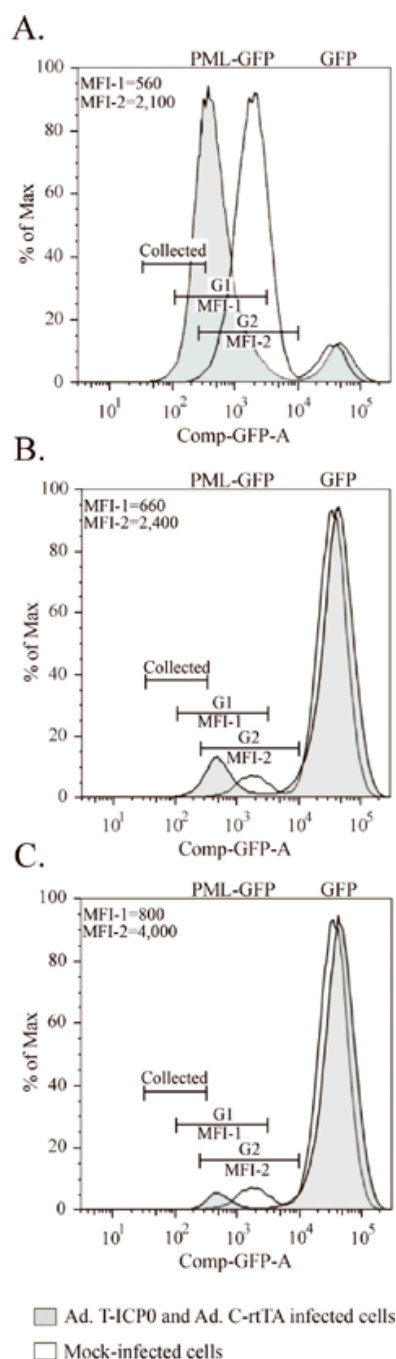


Figure 4. ICP0 reduces PML-GFP fluorescence in mixed cell populations as measured by flow cytometry

WI-38+PML-GFP and WI-38+GFP cells were trypsinized and mixed at ratios of 90% PML-GFP:10% GFP, 10% PML-GFP:90% GFP, and 1% PML-GFP:99% GFP. Twenty-four h post-plating, cells were mock-infected or infected with 8 or 20 PFU per cell Ad.T-ICP0 and Ad.C-rtTA in the presence of Dox. Twenty-four h post infection, cells were trypsinized and sorted by FACS. Graphs were then overlaid to show the fluorescence shift between infected (gray) and mock-infected (white) populations of A. 90% PML-GFP:10% GFP, B. 10% PML-GFP:90% GFP, and C. 1% PML-GFP:99% GFP. Gate 1 (G1) was used to determine the mean fluorescence intensity (MFI) of the infected WI-38+PML-GFP cells,

and gate 2 (G2) was used to determine the MFI of the mock-infected WI-38+PML-GFP cells. The “collected” gate was used to isolate infected cells with a fluorescence signal less than mock-infected WI+PML-GFP cells, which were further analyzed in Fig. 5. 100% of Max in A. represents 6,200-infected and 1,020-mock-infected cells, in B. 6,200-infected and 1,260-mock-infected cells, and in C. 6,650-infected and 1,270-mock-infected cells. A minimum of 50,000 events was scored for each sample. The experiment was repeated twice, and results from one experiment are shown.

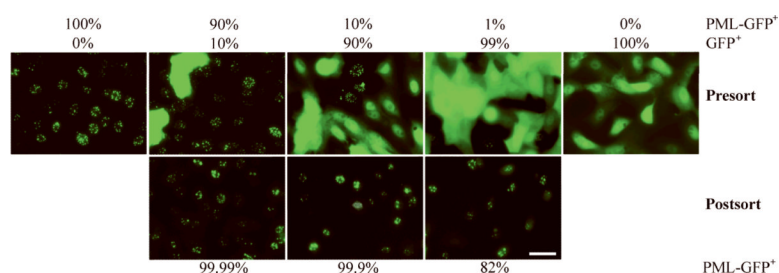


Figure 5. PML-GFP-expressing cells are selected in PML-GFP/GFP-mixed cell populations in the presence of ICP0

Presort (top row). WI-38 cells containing different percentages of PML-GFP and GFP prior to the addition of ICP0. *Postsort* (bottom row). Mixed cell populations from the presort were infected with 8 or 20 PFU per cell of Ad.T-ICP0 and Ad.C-rtTA for 24 h in the presence of Dox. Twenty-four h post infection, cells were trypsinized into a single cell suspension, and isolated cell populations from the collected gates in Fig. 4 were cultured on glass bottom plates in the absence of Dox for an additional 24 h. Cells were examined by fluorescence microscopy and photographed with a digital camera. The experiment was repeated twice, and results from one experiment are shown. The white bar at the bottom right is 50 μ m.